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Original Article

Yokukansan, a Kampo medicine, prevents the development of morphine tolerance through the inhibition of spinal glial cell activation in rats

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ABSTRACT

Background: Animal models have shown that glial cells (microglia and astrocytes) in the spinal cord undergo activation following peripheral injury associated with chronic pain, suggesting the involvement of these cells in pain diseases. We have previously reported that Yokukansan (YKS), a Japanese traditional herbal (Kampo) medicine, is effective against chronic pain through the suppression of spinal glial cell activation. Morphine is a widely-used opioid analgesic for relieving severe pain, but its repeated administration leads to the development of antinociceptive tolerance. The development of morphine tolerance is also reported to be caused by spinal glial cells activation. In the present study, we investigated the inhibitory effects of YKS on the development of morphine tolerance and the activation of the spinal microglia and astrocytes using a rat model.

Methods: Male Wistar rats received a subcutaneous injection of morphine hydrochloride (10 mg/kg/d) for 7 days, and the withdrawal latency to thermal stimulation was measured daily using a hot plate test. Thereafter, the appearance of activated microglia and astrocyte in the spinal cord (L5) was examined by immunofluorescence staining. Ionized calcium binding adapter molecule-1 (Iba-1) staining was used to label microglia and glial fibrillary acidic protein (GFAP) staining was performed to label astrocytes. YKS was administered mixed with powdered rodent chow at a concentration of 3%.

Results: The preadministration of YKS (started 3 d before the morphine injection) prevented the development of morphine tolerance. The repeated administration of morphine increased Iba-1 and GFAP immune reactivities in the spinal cord; however, these activations were inhibited by the preadministration of YKS.

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Conclusion: These results suggest that the preadministration of YKS attenuates the development of antinociceptive morphine tolerance, and the suppression of spinal glial cell activation may be one mechanism underlying this phenomenon.

1. Introduction

Glial cells are known to release various inflammatory cytokines and neurotrophic factors, such as adenosine triphosphate, interleukin (IL) -6 and -1β, tumor necrosis factor (TNF)-α, and nitric oxide, that lead to the regulation of neuronal functions and synaptic contacts.[1] Studies using animal models have reported that the glial cells (microglia and astrocytes) in the spinal cord undergo structural and functional modifications following peripheral injuries associated with chronic pain, suggesting the involvement of these cells in pain.[2,3] Therefore, these cells may potentially serve as targets for pain therapy.

Morphine is an opioid analgesic widely used for relieving severe pain such as that associated with cancer and surgery. However, its repeated administration may lead to the development of antinociceptive tolerance through the activation of spinal glial cells[4]; the administration of glial modulators, such as propentofylline, minocycline, and P2X4 receptor antisense oligonucleotide, may help attenuate this.[5,6]

Yokukansan (Yi-Gan San; YKS), first reported in the *Baoying jin-jing-lu* (written in 1550), is a traditional herbal (Kampo) medicine consisting of seven herbs (Table 1).[7] YKS has been administered to patients who show symptoms such as emotional irritability, neurosis, and insomnia and to infants who suffer from night crying and convulsions.[8] However, YKS has also recently been reported to be effective against pain disorders, such as headache, post-herpetic neuralgia, fibromyalgia, and trigeminal neuralgia.[9,10] Previous studies have demonstrated the antinociceptive effects of YKS in mice models with visceral pain[11] and rat models with chronic constriction injury.[12,13] Moreover, Nakagawa et al[14] reported that the preadministration of YKS daily for 3 weeks attenuates morphine tolerance; however, they did not discuss the mechanism involved sufficiently. We have previously reported that YKS has analgesic effects on chronic inflammatory pain using rat models with adjuvant arthritis, and one of the mechanisms involved was the inhibitory effect of YKS on the activation of microglial cells.[15]

Therefore, in the present study we investigated the inhibitory effect of YKS on the activation of spinal microglia and astrocytes using a rat model with morphine tolerance.

Table 1 – Component galenicals of Yokukansan (YKS; TJ-54)

Component galenicals of Yokukansan (YKS; TJ-54)	
Uncariae cum Uncis ramulus	3.0 g
Cnidii rhizoma	3.0 g
Bupleuri radix	2.0 g
Atratylodis Lanceae rhizoma	4.0 g
Poria	4.0 g
Angelicae radix	3.0 g
Glycyrrhizae radix	1.5 g

The weights show the mixing ratio.

2. Methods

2.1. Animals

This study used male Wistar rats (7 wk old, weighing 190–220 g) that were purchased from Nippon Bio-Supp. Center (Tokyo, Japan). During the study period, the animals were housed in standard plastic cages in our animal facilities at 25 ± 2 °C, 55 ± 5% humidity, and a 12-hour light/dark cycle. Food and water were provided *ad libitum*. All experiments were performed according to the guidelines of the Committee of Animal Care and Welfare of Showa University (certificate number: 02028).

2.2. Administration of YKS

YKS (Lot No. 2110054010; manufactured by Tsumura, Tokyo, Japan; Table 1) was mixed with powdered rodent chow (CE-2; CLEA Japan, Tokyo, Japan) at a concentration of 3% and fed to the YKS-treated rats. The rats that were not treated with YKS were fed powdered chow only. The concentration was chosen based on effective doses of YKS recommended by previous reports.[15]

2.3. Antinociceptive effect of YKS

The rats were randomly divided into a control group (Con; $n = 7$) and a YKS-treated group (YKS; $n = 7$), and thermal hyperalgesia was assessed daily for 10 days by measuring the withdrawal latency using a hot plate test.[16] The rats were placed on a hot plate (Hot plate analgesia meter model 39D; IITC Life Science, Woodland Hills, CA, USA) with the temperature adjusted to 47.5 °C. The latency up to the first sign of paw licking or jumping in response to the heat was measured, and 20 seconds was considered as the cutoff point to avoid tissue damage. The administration of YKS was started immediately after conducting the hot plate test on Day 1.

2.4. Influence of YKS on the antinociceptive effect of morphine

This test was performed to investigate whether YKS had any influence on the antinociceptive effect of a single morphine administration. The rats were randomly divided into a

pre-YKS + Mor group ($n = 9$) and a Mor group ($n = 9$). The pre-YKS + Mor group had been administered with YKS 3 days prior to the test. Each rat received a subcutaneous injection of morphine hydrochloride (10 mg/kg; Takeda Chemical Industries, Osaka, Japan), and the withdrawal latency was assessed using a hot plate test. The measurement was performed prior to and 30 minutes, 60 minutes, 90 minutes, 120 minutes, and 180 minutes after the administration of morphine.

2.5. *Inhibitory effect of YKS on morphine tolerance*

This test was performed to investigate whether YKS could inhibit the development of antinociceptive tolerance to morphine. The rats were randomly divided into a pre-YKS + Mor group ($n = 9$), a YKS group ($n = 9$), and a Mor group ($n = 9$). Each rat received a subcutaneous injection of morphine hydrochloride (10 mg/kg) once daily, and the withdrawal latency was measured after 30 minutes. The administration of YKS was started 3 days prior to morphine injection in the pre-YKS + Mor group and immediately after the first morphine injection in the YKS group.

2.6. *Changes in spinal microglia and astrocytes*

The appearance of activated microglia and astrocytes in the spinal cord was investigated using immunofluorescence staining. Ionized calcium binding adapter molecule-1 (Iba-1) staining was used to label microglia, and glial fibrillary acidic protein (GFAP) staining was used to label astrocytes.[6,13] The rats were randomly divided into the control group ($n = 5$), pre-YKS + Mor group ($n = 5$), and Mor group ($n = 5$). The control group was subcutaneously injected with physiological saline, whereas the pre-YKS + Mor and Mor groups were injected with morphine hydrochloride (10 mg/kg) once daily for 5 days. On Day 5, the rats were intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg; Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) 30 minutes after the morphine or saline administration and were intracardially perfused with phosphate buffered saline (PBS) at pH 7.4 until all of the blood had been removed from the system. After perfusion with 4% paraformaldehyde in 0.1 M PBS, the fifth lumbar spinal cords (L5) were harvested. Tissue specimens were embedded in optimal cutting temperature (OCT) compound, frozen and then cut into 30 μm sections using a cryostat (CM3050S; Leica Biosystems, Nussloch, Germany). The sections for microglia staining were first incubated overnight at 4 °C with rabbit anti-Iba-1 (1:500; Wako Pure Chemical Industries, Osaka, Japan) primary antibody and then incubated in fluorescein isothiocyanate (FITC)-conjugated goat Anti-Rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour at room temperature. The sections for the astrocyte staining were incubated overnight at 4 °C with mouse anti-GFAP-Cy3 antibody (C9205; 1:500, Sigma-Aldrich, St. Louis, MO, USA). The samples were observed using a confocal laser scanning fluorescence microscope (FV1000D, Olympus, Tokyo, Japan), and the optical densities of immunoreactive staining were measured using an appropriate software program (FV10-AW, Olympus). All values were reported as an average of ten micrographs per rat.

2.7. *Statistical analysis*

The experimental data are shown as the mean ± standard error of the mean (SEM). Significant differences were evaluated using a one-way analysis of variance test using SPSS for Windows (ver. 12.0) (IBM Corporation, Armonk, NY, USA). Comparisons between the three groups were performed using the *post hoc* Tukey test, whereas the Student *t* test was used for comparisons between the two groups. A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. *Antinociceptive effect of YKS upon thermal stimulation*

YKS was administered for 10 days, and the withdrawal latency was measured daily. No significant differences were observed between the two groups (Fig. 1). Thus, YKS did not increase the withdrawal threshold value in intact rats.

3.2. *Influence of YKS on antinociceptive effects of morphine*

The influence of YKS on the antinociceptive effects of a single morphine administration was examined, and the withdrawal latencies were observed to increase after 120 minutes in both groups and return to the same level as before administration, after 180 minutes (Fig. 2). No significant differences were observed between the two groups during this period. Therefore, YKS did not prolong the reaction duration of morphine.

3.3. *Inhibitory effect of YKS on morphine tolerance*

We also examined if the administration of YKS had any influence on the antinociceptive effect of daily morphine administration (Fig. 3). On Day 3, the withdrawal latencies significantly decreased in the Mor (10.94 ± 4.01 s) and YKS + Mor (10.98 ± 2.50 s) groups compared with withdrawal latency in the pre-YKS + Mor group (18.90 ± 1.10 s; $p < 0.01$). This

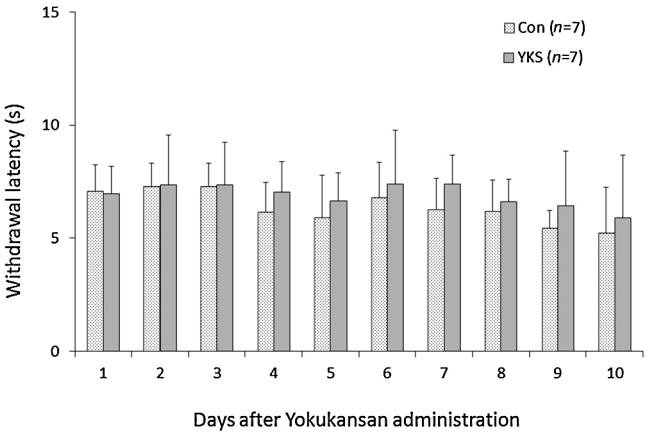

Fig. 1 – Antinociceptive effect of Yokukansan (hot plate test). Yokukansan was administered for 10 days, and it did not increase the withdrawal latency in intact rats. Con, control group; YKS, Yokukansan-treated group.

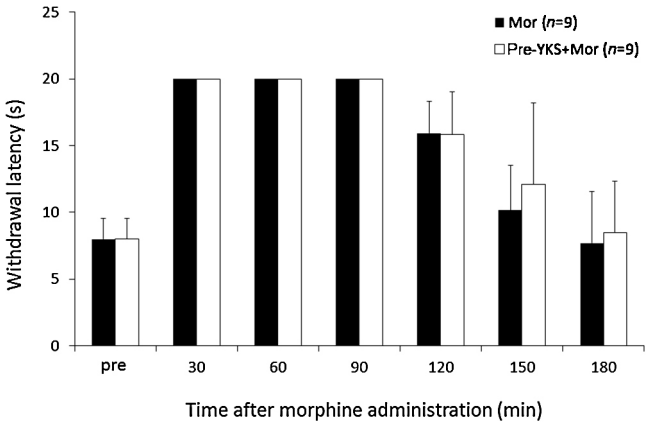

Fig. 2 – Influence of Yokukansan on antinociceptive effect of morphine (hot plate test). Reaction duration of morphine lasted for 120 minutes, and Yokukansan did not have any influence on the antinociceptive effect of morphine. Mor, morphine-treated group; pre-YKS + Mor, Yokukansan-pre-treated + morphine-treated group.

significant difference persisted even on Day 7 (the Mor group: 6.41 ± 1.45 s, the YKS + Mor group: 5.34 ± 0.81 s, the pre-YKS + Mor group: 9.15 ± 3.36 s; $p < 0.05$). Therefore, preadministration may help prevent the development of morphine tolerance.

3.4. *Changes in spinal glial cells*

3.4.1. *Changes in spinal microglia*

The Iba-1 immunoreactivity was assessed in the spinal cord (L5) (Fig. 4), and the images of the spinal dorsal horns are shown in Fig. 4A–4F. The number of Iba-1-positive cells in the Mor group increased (Fig. 4B) in comparison with that in the control group (Fig. 4A) and the pre-YKS + Mor group (Fig. 4C; 200× magnification). The Iba-1 values were then expressed as optical densities (Fig. 4G). Ten images (at 2 μm intervals) were recorded per rat, the immune reactivities were quantified, and all the values were reported as an average of ten micrographs per rat. The Iba-1 level was significantly increased in the Mor group (231.1 ± 28.2 immunoreactivity (IR) density) compared with that in the control group (143.0 ± 59.3 IR density; $p < 0.05$). This increase was significantly inhibited in the pre-YKS + Mor group (151.3 ± 44.0 IR density; $p < 0.05$). Furthermore, microgliosis was confirmed in the image (600×) of the Mor group (Fig. 4E) based on intense Iba-1 immunoreactivity, large cell bodies, and short or thick processes of microglia. However, this change was suppressed in the pre-YKS + Mor group (Fig. 4F).

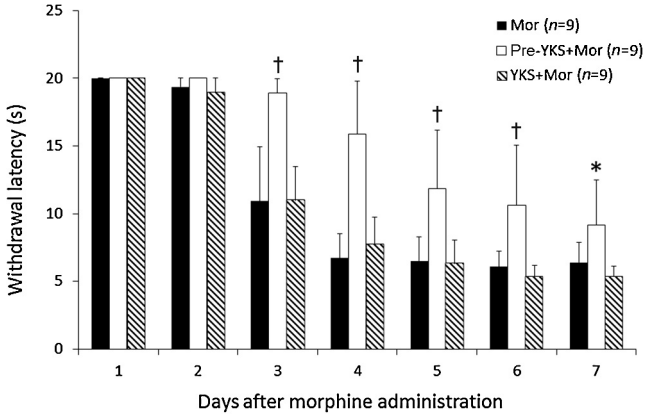

Fig. 3 – Inhibitory effect of Yokukansan (YKS) on morphine tolerance. From Day 3 to Day 7, the decrease in withdrawal latency was significantly inhibited in the pre-YKS + Mor group in comparison with that in the Mor and YKS + Mor groups (Days 3–6: $p < 0.01$, Day 7: $p < 0.05$). Preadministration could prevent the development of morphine tolerance.
*** $p < 0.05$ (vs. Mor, YKS + Mor).**
† $p < 0.01$ (vs. Mor, YKS + Mor).
Mor, morphine-treated group; pre-YKS + Mor, Yokukansan-pretreated + morphine-treated group; YKS + Mor, Yokukansan-treated + morphine-treated group.

3.4.2. *Changes in spinal astrocytes*

The GFAP immunoreactivity was assessed in the spinal cord (L5; Fig. 5), and the images of the spinal dorsal horns are shown in Fig. 5A–F. The Mor group showed an increase in the number of GFAP-positive cells (Fig. 5B) when compared with the control group (Fig. 5A) and the pre-YKS + Mor group (Fig. 5C; 200× magnification). The GFAP values were then expressed as optical densities (Fig. 5G). The GFAP level was significantly increased in the Mor group (714.8 ± 255.7 IR density) compared with that in the control group (228.1 ± 45.5 IR density; $p < 0.05$). This increase was significantly inhibited in the pre-YKS + Mor group (326.0 ± 104.2 IR density; $p < 0.05$). Furthermore, astrogliosis was confirmed in the image (600×) of the Mor group (Fig. 5E) based on intense GFAP immunoreactivity and hypertrophied astrocytes with thick processes. However, this change was suppressed in the pre-YKS + Mor group (Fig. 5F).

4. Discussion

The present study indicates that the administration of YKS does not have any apparent antinociceptive effects and does not prolong the reaction duration of morphine. However, the preadministration of YKS may inhibit the development of antinociceptive tolerance to morphine. It is believed that YKS prevents a decrease in pain threshold by improving pathologic changes, although YKS does not increase withdrawal latency in the physiological state. Nakagawa et al[14] reported that the repeated preadministration of YKS for 3 weeks prevented the development of morphine tolerance and physical dependence in mice. In this study, preadministration for 3 days affected the development of morphine tolerance, although no such effects could be seen in rats simultaneously administered with YKS and morphine. The reason for this is still unclear, and further studies are warranted.

Morphine tolerance is a complex physiological response involving glial cell activity,[4–6] neuroinflammation,[17,18] and the activation of spinal N-Methyl-D-Aspartate (NMDA) receptors.[19] Many studies report that glial cells may be involved in the development of antinociceptive tolerance to morphine, and glial activation inhibitors (such as P2X4 receptor antagonist, P2X4 receptor antisense, minocycline,

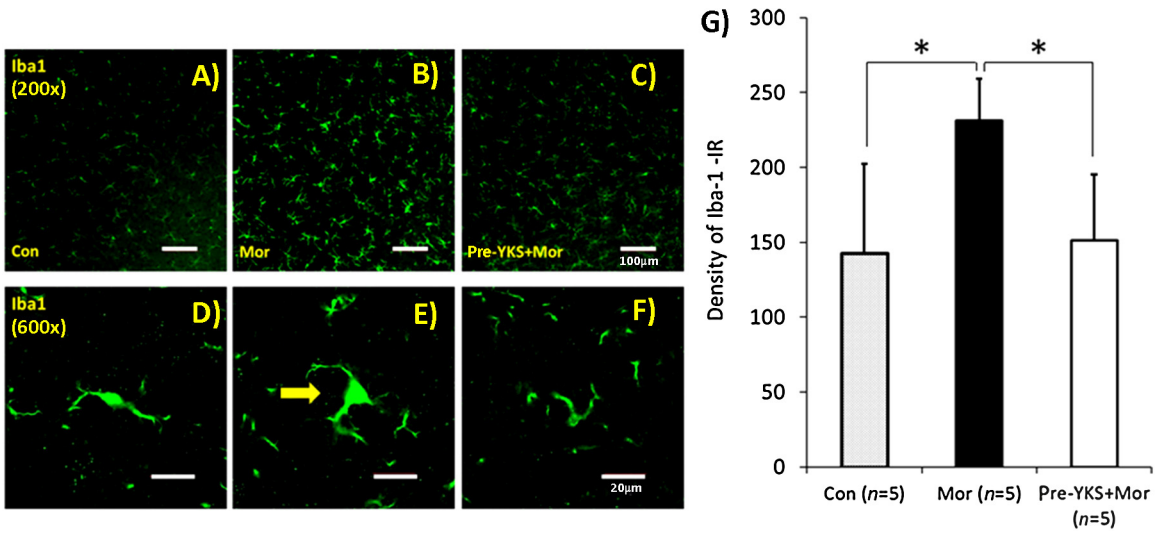

Fig. 4 – Inhibitory effect of Yokukansan (YKS) on microglia activation induced by morphine treatment. (A–C) 200× magnification; scale bar = 100 μm. (D–F) 600× magnification; scale bar = 20 μm. (A–F) Ionized calcium binding adapter molecule-1 (Iba-1) staining of the L5 spinal dorsal horn on Day 5 following morphine or saline treatment, and (G) the fluorescence density quantification of Iba-1 immune reactivity. Iba-1 expression in (B) the Mor group increased significantly compared with that in (A) the control group; however, this increase was inhibited in (C) the pre-YKS + Mor group. (E) In the Mor group, microgliosis (yellow arrow) was confirmed based on intense Iba-1 immunoreactivity, large cell bodies, and the short or thick processes of microglia; however, this change was suppressed in (F) the pre-YKS + Mor group.
*** $p < 0.05$.**
Con, control group; Iba-1, ionized calcium binding adapter molecule-1; IR, immunoreactivity; Mor, morphine-treated group; pre-YKS + Mor, Yokukansan-pre-treated + morphine-treated group.

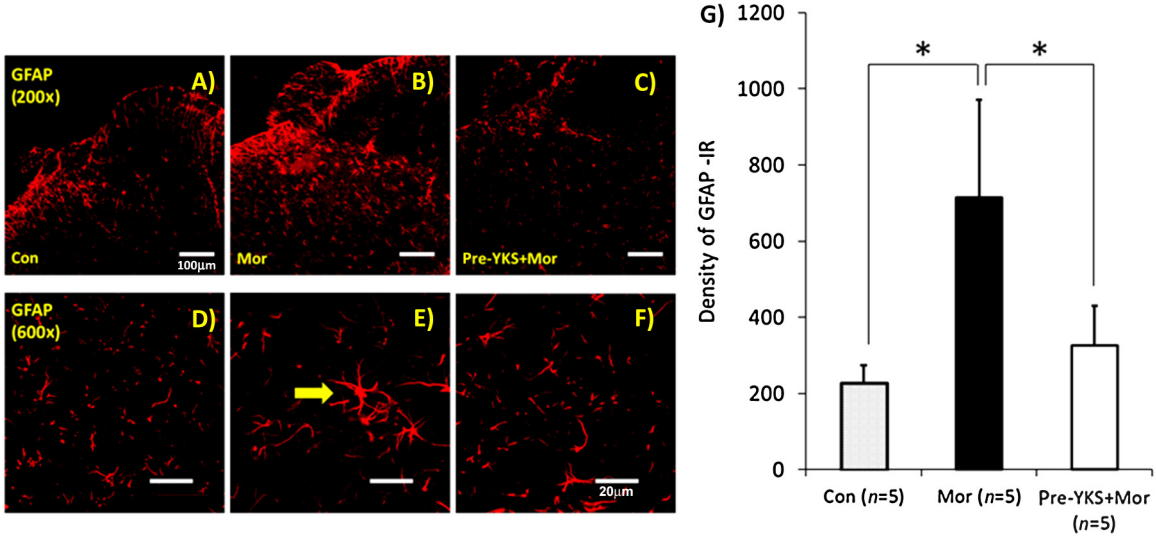

Fig. 5 – Inhibitory effect of Yokukansan on astrocyte activation induced by morphine treatment. (A–C) 200× magnification; scale bar = 100 μm. (D–F) 600× magnification; scale bar = 20 μm. (A–F) Glial fibrillary acidic protein (GFAP) staining of the L5 spinal dorsal horn on Day 5 following morphine or saline treatment, and (G) the fluorescence density quantification of GFAP immune reactivity. GFAP expression in (B) the Mor group increased significantly compared with that in (A) the control group; however, this increase was inhibited in (C) the pre-YKS + Mor group. (E) In the Mor group, astrogliosis (yellow arrow) was confirmed based on intense GFAP immunoreactivity and hypertrophied astrocytes with thick processes; however, this change was suppressed in (F) the pre-YKS + Mor group.
*** $p < 0.05$.**
Con, control group; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; Mor, morphine-treated group; pre-YKS + Mor, Yokukansan-pre-treated + morphine-treated group.

propentofylline, and pentoxifylline) delay the development of this tolerance.[5,6] Pentoxifylline is known to be a cytokine inhibitor that decreases the levels of TNF-α, IL-1, and IL-6.[20] A clinical study reported that pentoxifylline enhances the effects of morphine after surgery, and patients who received preoperative pentoxifylline had lower opioid requirements and serum levels of IL-6 compared with those in the control group.[21] Ebisawa et al[13] reported that YKS improves mechanical allodynia through the regulation of IL-6 expression in microglia and astrocytes in the spinal cord.

The activation of the spinal NMDA receptor, a glutamate receptor found in nerve cells, also plays a crucial role in the development of morphine tolerance,[19] and a pharmacological blockade of these receptors may attenuate this.[22] Moreover, the activation of central NMDA receptors is involved in microglial activation, and they can be inhibited by MK-801 (an NMDA receptor antagonist).[23] Kawakami et al[24] reported that YKS includes isoliquiritigenin, which is known to act as an NMDA receptor antagonist. In this study, immune reactivities of Iba-1, a marker of microglia, and GFAP, a marker of astrocytes, increased with the development of morphine tolerance. This was in accordance with past reports.[4–6] However, these increases were controlled by the preadministration of YKS. The inhibitory effects of YKS on IL-6 expression and the antagonistic action of NMDA receptors may be responsible for this. We will investigate the role of IL-6 and NMDA receptors in the effects of YKS on antinociceptive tolerance to morphine in the future.

To the best our knowledge, this study is the first to examine the relationship between YKS and glial cell activation in morphine tolerance. The combined administration of YKS and morphine may attenuate morphine tolerance. Several other studies have recently been conducted to clarify the pharmacological actions of YKS, and they reported an ameliorative effect on glutamate clearance in astrocytes.[25] In the serotonin-responsive nervous system, YKS has a partial agonistic effect on the serotonin 1A receptor, which is associated with analgesia,[26] and a downregulatory effect on the serotonin 2A receptor, which is associated with pain.[27] Further studies are needed to understand the mechanism of the effects of YKS on antinociceptive tolerance to morphine so as to allow its effective use in patients.

This study suggests that the preadministration of YKS attenuates the development of antinociceptive morphine tolerance, and the suppression of spinal glial cell activation may be one mechanism underlying this phenomenon.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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